

Isolation and Identification of Three-Rings Polyaromatic Hydrocarbons (Anthracene and Phenanthrene) Degrading Bacteria

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Abstract: There are many industrial areas which produce large amount of poly-aromatic hydrocarbons (PAHs) in Egypt which represent severe hazards effects on the ambient environment. Bacterial strains were isolated from different contaminated sites in middle delta, Egypt and screened for PAHs degradation. Enrich media was used to isolate the anthracene and phenanthrene degrading bacteria. Fourteen bacterial isolates showed high degradation for both anthracene and phenanthrene. For genotyping, these isolates were subjected to RAPD-PCR using four different primers. The data showed that the fourteen isolates were not related to each other. Only four isolates showed the highest ability for degradation were subjected to 16S rDNA sequence for identification. Partial sequence of 16S rDNA revealed that these isolates were *Escherichia coli* (EF105548), *Soil bacterium* (EF105549), *Alcaligenes sp.* (EF105546) and *Thiobacter subterraneus* (EF105547). The average degradation rates of anthracene by *Escherichia coli* (EF105548), *Soil bacterium* (EF105549), *Alcaligenes sp.* (EF105546) and *Thiobacter subterraneus* (EF105547) were 28.57, 30.19, 26.5875 and 32.11%, while those of phenanthrene were 42.45, 48.44, 34.35 and 40.45% for these strains, respectively.

Key words: PAHs % Anthracene % Phenanthrene % RAPD-PCR % 16S rDNA % *E. coli* % *Soil bacterium* % *Alcaligenes sp.* % *Thiobacter subterraneus*

INTRODUCTION

Environmental pollution was increased by increasing the industry development all over the world and especially in Egypt; increment of these pollution caused many hazards for all organisms, even for humans such as carcinogenicity and toxicity. Also there has been increasing pollution with hydrocarbon compounds, many of these hydrocarbons considered to be a potential health hazard [1, 2]. Some of hydrocarbon compounds pollutants are polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAHs) are a large group of

organic compounds with two or more fused aromatic rings in linear, angular, or cluster arrangements. They have a relatively low solubility in water, but are highly lipophilic [2, 3]. Most of the PAHs with low vapor pressure in the air are adsorbed on particles. When dissolved in water or adsorbed on particulate matter, PAHs can undergo photodecomposition when exposed to ultraviolet light from solar radiation. In the atmosphere, PAHs can react with pollutants such as ozone, nitrogen oxides and sulfur dioxide, yielding diones, nitro- and dinitro-PAHs and sulfonic acids, respectively. PAHs may also be degraded by some microorganisms in the soil [2, 4].

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Although PAHs can exist in over 100 different combinations, the most common are treated as a group of 15 they are: acenaphthene, acenaphthylene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, Fluoranthene, Fluorine, indeno(1,2,3cd)pyrene, phenanthrene, pyrene, [5, 6]. Anthracene and phenanthrene are tri-cyclic aromatic hydrocarbons that are found in high concentrations in polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils and waste sites. These hydrophobic contaminants are widely distributed in the environment, occurring as natural constituents of fossil fuels and their anthropogenic pyrolysis products [2, 7, 8, 9]. Unlike the higher-molecular-weight PAHs, phenanthrene and anthracene do not pose a risk to human health, since they exhibit no genotoxic or carcinogenic effects. However, they have been shown to be toxic to fish and algae [10, 11].

Most of the PAHs are used to conduct research. However, some of the PAHs are used to make dyes, plastics; detergent, fungicides and pesticides. Some are even used in medicines. One of the most common ways PAHs can enter the body is through breathing contaminated air. The PAHs get into the human lungs when they breathe them. In addition if the human eat or drink food and water that are contaminated with PAHs, they could be exposed. Exposure to PAHs can also occur if the skin touches PAH contaminated soil or products like heavy oils, coal tar, roofing tar or creosote [12]. Creosote is an oily liquid found in coal tar and is used to preserve wood. Once the PAHs enter the human body, they can spread and target fat tissues. Target organs include kidneys, liver and fat. However, in just a matter of days, the PAHs will leave the human body through urine and feces [12].

A variety of bacterial species have been isolated that have the ability to utilize anthracene or phenanthrene as the sole source of carbon and energy [7, 13, 14]. The initial reactions in the degradation of anthracene and phenanthrene are catalyzed by multicomponent dioxygenases that incorporate both atoms of molecular oxygen into the PAH nucleus to produce *cis*-dihydrodiols [15, 16]. Genes involved in PAH metabolism and its regulation have been described for *Pseudomonas*, *Sphingomonas* and *Nocardioides* species [2, 17- 22].

In Egypt there are many of industrial areas which produce large amount of polyaromatic hydrocarbons (PAHs). Kafr-Zaiat is one of these areas, which has many

of contamination sources due to existence of several industrial complex concerning with Paper, Pesticide, Petrochemical and oil refinery. This location is in the middle delta of Egypt, where there are intensive urban and ruler population, which expose the ambient environment, human health and aquatic fauna to severe hazards effects. Therefore, there is urgent need to remove this contaminant from the environment. One of the most safe and efficient way to execute this issue is to bioremediation of these contaminants using selected microorganisms. Therefore, the main objective of this study was to discover and isolation of the bacterial strains which able to degrade such pollutants as anthracene and phenanthrene from the Egyptian soil. Meanwhile, upgrade of these organisms to be highly degrading for such compounds.

MATERIALS AND METHODS

Soil Sample Collections: Soil and water samples were collected from different contaminated sites (paper company drainage, Tala drainage, Housing drainage, Pesticide Kafr-Zaiat company drainage, Petrochemical industries and oil refinery) containing a complex mixture of polyaromatic hydrocarbons (PAHs), including anthracene, phenanthrene and other PAHs.

Bacterial Isolation: Soil samples (0.1 g) were added into 50 ml mineral salt medium [23]. The medium containing the soil and PAHs was incubated at $30\pm 2^{\circ}\text{C}$ under shaking (200 rpm) for 24 h. Bacterial enrichment cultures were set up in 500 ml cotton-plugged Erlenmeyer flasks containing anthracene or phenanthrene as a sole source of carbon. Contaminated stock solution of anthracene and phenanthrene (20 mg/ml) were prepared in dichloromethane and added to the mineral salt medium at an initial concentration of 10 mg/l. The anthracene and phenanthrene concentration in the mineral salt medium were slowly increased up to 500 mg/l over a period of 57 days and were maintained at this level for the duration of the enrichment at 30°C with shaking 200 rpm. After one weeks of enrichment, sub-samples from the flasks were streaked on plates containing solid medium. Plates were prepared with MSM that had been solidified with 2% agar and sprayed with a 20 Mg/l anthracene and phenanthrene solution dissolved in dichloromethane. The plates were incubated at 30°C temperature. The colonies were grown on the plates were picked and streaked on new minimal agar plates.

Degradation of Anthracene and Phenanthrene: The degradation of anthracene and phenanthrene was examined using the real time method [24]. Comparison between isolates that able to degrade anthracene and phenanthrene were carried out using flourescan apparatus (Labsystem, Finland). In 96 well ELISA plate, 7.5 µl of 4% anthracene or phenanthrene concentration (dissolved in dichloromethane), 15µl of isolate culture and 277.5 µl MSM were added (total volume in each well 300 µl), the plate was incubated in flourescan apparatus for 24 h which optimize on plate template 96-well costar 3596, temperature 30°C, speed 200 rpm, measure, interval 1 h, measure count 24 h, filter pair excitation 355 and emission 460 nm. UV light is required to excite the emission of visible light, when UV light is passed through a sample; the sample emits light (fluorescence) proportional to the concentration of the anthracene or phenanthrene.

DNA Extraction and PCR Amplification Conditions:

RAPD-PCR was carried out using four primers: BOXA1R primer(5`-CTACGGCAAGGCGACGCTGACG-`3)[25,26], REP1R-I primer (5`-CGGICTACIGCIGCIII-`3) [25], chitinase reverse primer (5`-GGAGCCAGGCGGTCCARTCGGGCCACCA-`3)and R2 primer(5`-GGAGCCAGGCGGTCCARTCGGGCCACCA-`3). PCR mixtures were prepared with 1µl of genomic DNA, 5 µl of PCR buffer 10X, 2.5 mM of MgCl₂, 200 µmol of each deoxynucleoside triphosphate, 20 pmol each primer, 5 µg of bovine serum albumin, 1% of formamide and 2.5 U *Taq* polymerase (Promega, Germany) and sterile filtered mille water to a final volume of 50 µl. PCR amplification was as follows: denaturation at 95°C for 5min, 40 cycles was applies as follow: 94°C for 30s, 35°C for 30s and 72°C for 1 min in final extension step at 72°C for 7 min. PCR products were separated on 1.5% agarose gel electrophoresis. Size of fragments were estimated by using DNA marker (100 base-pair ladder). The gel was photographed by using Gel Doc System. The phylogeny tree of bacterial isolates was analyzed using Statistica V 5 software. The highly anthracene- and phenanthrene-degradable isolates (the data are not showed) and relatively distance isolates according to the results in the dendrogram (Fig. 2) were selected and subjected to sequence analysis of 16S rDNA genes.

Sequence Analysis of 16S rDNA Genes: Approximately 350 bp was selected from the polymorphic region of 16S rDNA gene and amplified using specific primers. PCR reaction was performed on the system 9700 thermocycler under the following conditions: 34 cycles of denaturation

at 94°C (1 min), annealing at 58°C (1 min) and extension at 72°C (1 min). A 350-bp product was amplified using the forward primer 5`-AACTGGAGGAAGGTGGGGAT-3`and reverse primer 5`-AGGAGGTGATCCAACCGCA-3` [27]. PCR product was analyzed in 2% agarose gel stained with ethidium bromide. Gels were photographed by Gel Doc system (Alpha Imager TM1220, Documentation and Analysis system, Canada).

Sequencing steps was performed at Gene Analysis unit, VACSERA. Cycle sequencing was done by using a big dye terminator cycle sequencing kit (Applied Biosystems, Foster City). Sequencing products were purified by using Centri-sep spin Column and were resolved on an applied Biosystems Model 310 automated genetic analyzer. Approximately 350 bp was sequenced and phylogenetic and molecular evolutionary analyses of the both anthracene and phenanthrene degrading bacteria based on 16S rDNA genes were conducted using *MEGA* version 4 [28].

RESULTS AND DISCUSSION

RAPD Fingerprinting of Bacterial Isolates DNA: All the isolated bacteria were subjected to RAPD-PCR using four primers: BOXA1R, REP1R-I, chitinase reverse primer and R2 (Fig. 1). The phylogeny tree (Fig. 2) obtained from statistical analysis of the RAPD-PCR band pattern (Fig. 1) showed that, there are only two main groups branched from one ancestor. These two groups branched in linkage distance 36% into five sub-groups and these five sub-groups are divided into 7 classes at distance linkage percent 30%. Whenever, at distance linkage 17% there is no similarity had been observed.

Alignments and Phylogenetic Analysis: The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in GenBank revealed that the isolates are similar to *E. coli* (EF105548) with 96% similarity and to *E. coli* (IF4 and AE1-2), *Uncultured bacterium* (EF105549) with 98%, *Uncultured bacterium* clones (JSC2-D5 and D1-52), *Alcaligenes sp.* (EF105546) with 95% similarity, *Alcaligenes sp.*OO4 and 100% similarity to strain *Alcaligenes faecalis*. Strain: ZJSO2 and the bacterial strain *Thiobacter subterraneus* with accession No EF105547 revealed 90% similarity to *Thiobacter subterraneus* as showed in Table 1.

Table 1: Similarity percentage of 16S rDNA sequences for the selected isolates compared to those obtained from database

| Isolate | Organism | Identity (%) | Accession No. |
|----------------------------------|---|--------------|---------------|
| <i>E.coli</i> | | | |
| accession No. EF105548 | <i>E.coli</i> .strain: IF4 | 96 | AB272358 |
| | <i>E.coli</i> .strain: AE1-2 | 96 | AB269763 |
| <i>Unculture soil bacteria</i> | | | |
| accession No. EF105549 | <i>Uncultured bacterium</i> clone JSC2-D5 | 98 | DQ532176 |
| | <i>Uncultured bacterium</i> clone D1-52 | 98 | DQ113754 |
| <i>Alcaligenes</i> sp. accession | | | |
| No EF105546 | <i>Alcaligenes</i> sp.OO4 | 95 | AB 272325 |
| | <i>Alcaligenes faecalis</i> strain zjs02 | 100 | DQ857898 |
| <i>Thiobacter subterraneus</i> | | | |
| accession No. EF105547 | <i>Thiobacter subterraneus</i> | 90 | AB180657 |

The phylogeny of the bacterial strains and closely related species was analyzed using the multi-sequence alignment program and the results are presented in Fig. 3. The dendrogram displayed that the four strains were grouped in one cluster, which is divided into two sub-clusters, one of them include strain *E. coli* with accession No. EF105548, while the other sub-cluster includes the remaining three strains. Consecutively this sub-cluster divided into two groups, one of these groups contains the three strains. Two of these strains (*Alcaligenes* sp. with accession no. EF105546 and *Thiobacter subterraneus* with accession no. EF105547) are closely related two each other in one sub-group, while the other strain (*Unculture soil bacteria* with accession No. EF105549) is distantly far and enclosed in a defined specific sub-group.

Degradation Rate of Anthracene and Phenanthrene: The degradation rate of anthracene and phenanthrene for the four selected strains (*Escherichia coli* EF105548, *Soil bacterium* EF105549, *Alcaligenes* sp. EF105546 and *Thiobacter subterraneus* EF105547) over 24 h is showed in Fig. 4A and B. The average degradation rates of anthracene by *Escherichia coli* (EF105548), *Soil bacterium* (EF105549), *Alcaligenes* sp. (EF105546) and *Thiobacter subterraneus* (EF105547) were 28.57, 30.19, 26.5875 and 32.11% (Fig. 4A), while those of phenanthrene were 42.45, 48.44, 34.35 and 40.45% (Fig. 4B) for these strains, respectively. In addition, it is obvious from Fig. 4A that the most efficient anthracene degrading strain was *Thiobacter subterraneus* (EF105547), while Fig. 4B indicated that the most phenanthrene degrading strain was *soil bacterium* (EF105549). In respect to degradation rate over time, it is obvious from Fig. 4A,B that degradation rate of both anthracene and phenanthrene increase gradually by

almost constant values over the 24 h of the degradation experiment for all of the strains.

The results of the present study confirmed the matter that many of bacterial strains, especially gram-negative bacteria were found to degrade poly aromatic hydrocarbons (PAHs) compounds at various extents [7, 13, 14]. Soil samples contaminated with PAHs were used to isolate bacteria capable of mineralizing several PAHs as sole carbon source. The isolated strains belong to genera known to exhibit special metabolic potential in soil environments [29]. *Pseudomonas* and *Alcaligenes* spp. are, besides *Acinetobacter* spp., the prevailing bacteria occurring at polluted sites due to enhanced selection by high concentrations of organic xenobiotics [30]. In this context, Abd-Elsalam *et al.* [25] isolated two strains of bacteria *Flavobacterium* sp. (DQ398100) and *Pseudomonas putida* (DQ399838) from the same contaminated sites of the present study. They reported that *Pseudomonas* sp. showed high ability to degrade about 50% of the total phenol and naphthalene in the medium after 20 h from inoculation, whenever the *Flavobacterium* strain degrades 50% of naphthalene in the same time while degraded the same amount of phenol in 17 h.

All of PAHs degrading bacterial strains which have been identified in the present study were gram negative, which agreed with the results indicated that most efficient of the PAHs degrading bacteria were belong to the genus *Pseudomonas* [7]. In this context, Coral and Karagoz [31] isolated two pseudomonas bacterial strains from petroleum refinery soil (ARP26 and ARP28) that were capable to degrade phenanthrene at rate of 93 and 98%, respectively within the first seven days of incubation. In agreement of the present results Somtrakoon *et al.* [32] found that addition of *Burkholderia* sp. VUN10013 (initial concentration of 105 cells g⁻¹ dry soil) to autoclaved soil

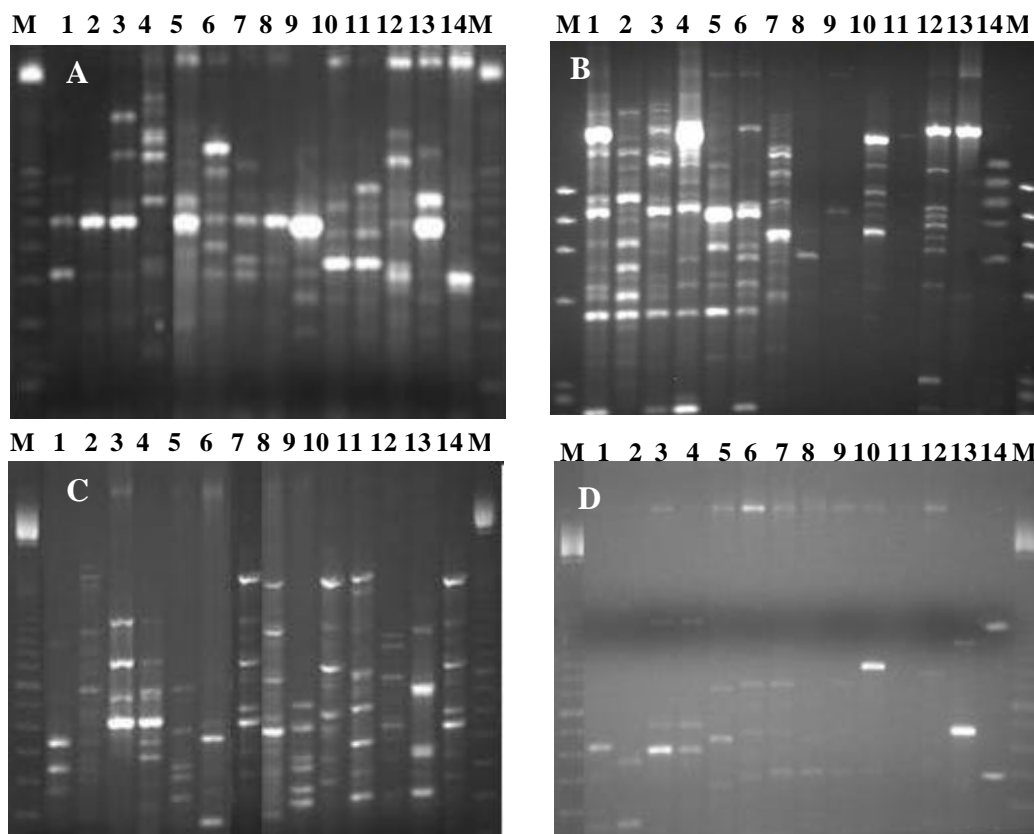


Fig. 1: RAPD-PCR for 14 bacteria isolates; A: using BOX1AR primer; B: using PRIM1 primer, C: Chitinase reverse primer; D, R2 primer. M= DNA marker with 100 base-pair ladder.

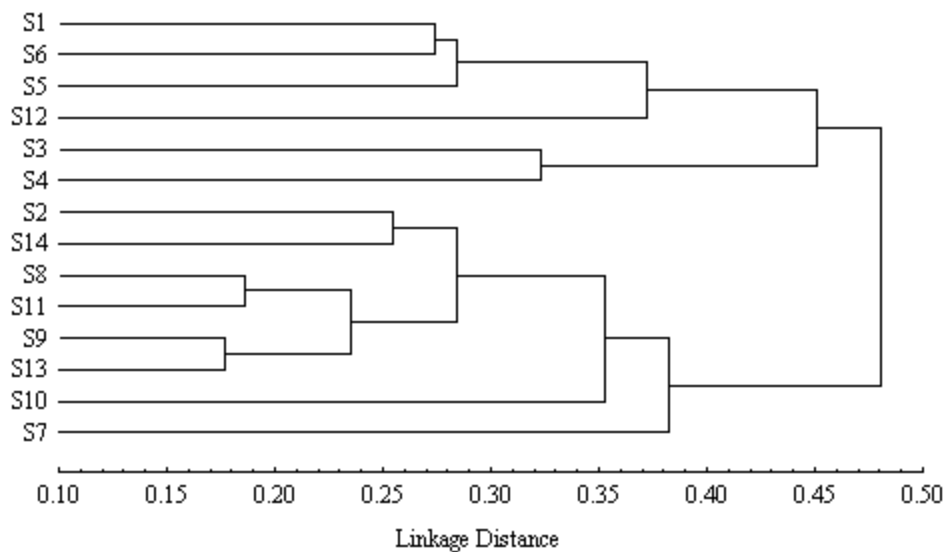


Fig. 2: Dendrogram of the bacterial isolates based on RAPD-PCR results

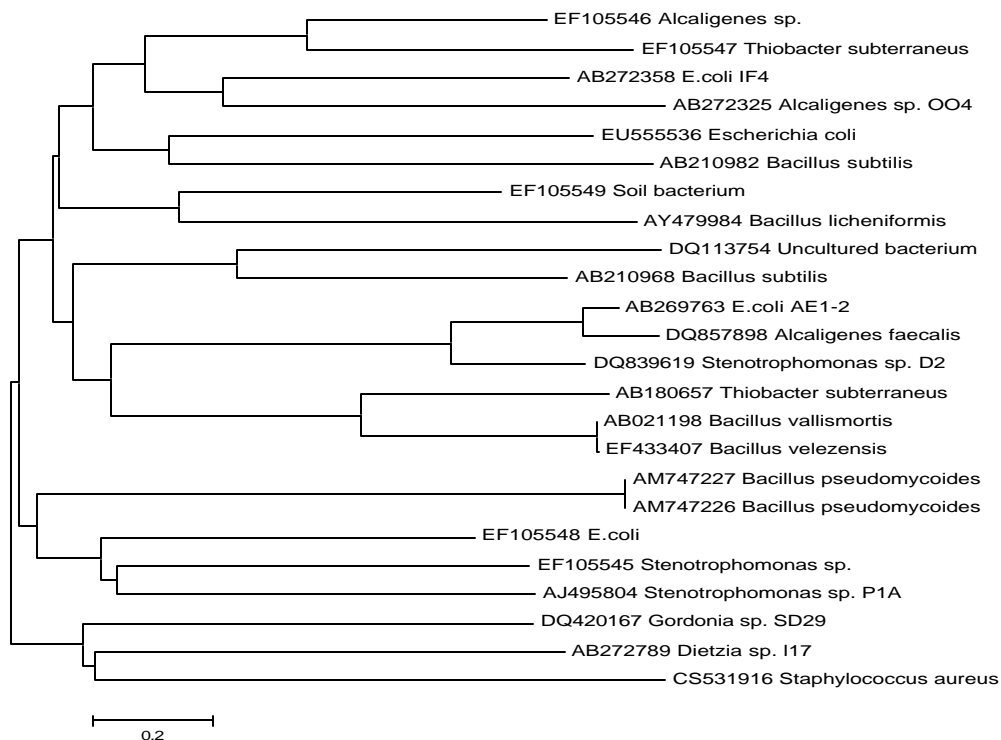


Fig. 3: Phylogenetic relationships among the selected strains based on sequence analysis and the most closely related bacteria species

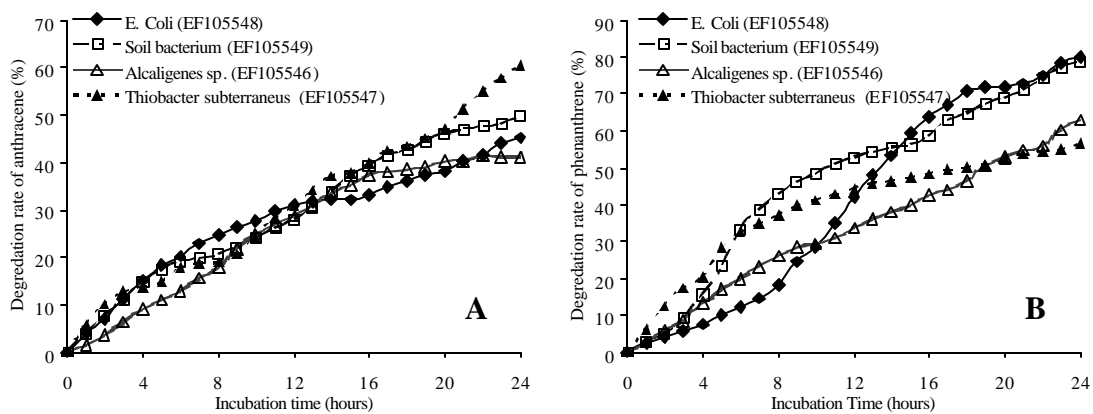


Fig. 4: The rates of degradation for anthracene and phenanthrene with different four bacterial isolates compared with incubation time (h)

collected from the Plew District, Chanthaburi Province, Thailand, supplemented with anthracene (50 mg anthracene kg⁻¹ dry soil) resulted in complete degradation of the added anthracene within 20 days. However, it should be mentioned that they used much less concentration of anthracene than it was used in the present study (4% of anthracene). The variation of results obtained by different studies may be interpreted on the basis of the variation of bacterial strains used, time of adaptation and incubation conditions. In an earlier study with genus *Alcaligenes* which have been proved to degrade PAHs in the current study, Walter *et al.* [33] indicated that *Alcaligenes denitrificans* isolated from soil using conventional enrichment techniques was able to degrade fluoranthene at the rate of 0.3 mg fluoranthene mlG⁻¹ per day at doubling times of 35 h for growth on PAH as sole carbon source. On the other hand, Moller and Ingvorsen [34] isolated *Alcaligenes sp.* from oil polluted soil and they reported that Addition of *Alcaligenes sp.* to soil microcosms supplemented with phenanthrene (1 mg phenanthrene gG⁻¹ dry soil) resulted in degradation of the added phenanthrene within 11 days. The phenanthrene concentration declined only 12% in uninoculated soil during 42 days. The total phenanthrene degradation potential of *Alcaligenes sp.* was 2.3 mg phenanthrene gG⁻¹ dry soil during a period of 22 days. The amount of CO₂ evolved during 22 days corresponded to the conversion of 91% of the degraded phenanthrene to CO₂.

CONCLUSION

Four native Egyptian anthracene and phenanthrene degrading bacterial strains were isolated from different contaminated soil and water sites. These bacterial strains were identified as *Escherichia coli* (EF105548), *Soil bacterium* (EF105549), *Alcaligenes sp.* (EF105546) and *Thiobacter subterraneus* (EF105547). The average degradation rates of anthracene by *Escherichia coli* (EF105548), *Soil bacterium* (EF105549), *Alcaligenes sp.* (EF105546) and *Thiobacter subterraneus* (EF105547) were 28.57, 30.19, 26.5875 and 32.11%, while those of phenanthrene were 42.45, 48.44, 34.35 and 40.45% for these strains, respectively. Additional studies have to be made to reveal the optimum conditions (nutrients, temperature, gaseous pressure, etc.) require to maximum anthracene and phenanthrene degradation by pure or mixed cultures of these strains.

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